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GENOTOXIC AND ANTI-MUTAGENIC EFFICACY OF PURIFIED ANTHOCYANIN FROM SELECTED *OSBECKIA* SPECIES ON CYCLOPHOSPHAMIDE INDUCED CHROMOSOME ABERRATIONS IN ALBINO MICE

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ABSTRACT

This study aims to evaluate the genotoxic and anti-mutagenic activity of purified anthocyanin from the species Osbeckia aspera and O. reticulata on mice. Anthocyanin was extracted optimally from the in vitro calli cultures of the species using the hormonal combination and doses of 2, 4-D, BA, NAA and subsequently, purified by column chromatography. The major fractions identified by LC-MS/MS were malvidin-3 -diglucoside, delphinidin, cyanindin aglycone and peonidin. Then, the purified anthocyanin extract was made into a suspension using 0.5% carboxy methylcellulose with 3 different concentrations (100, 200, and 400 mg/kg) and was administrated orally in mice for 7 consecutive days. 4 h after the last oral administration, cyclophosphamide (CP) 50 mg/kg IP was injected. 30 h after CP injection, the mice were sacrificed, and the samples of bone marrow were prepared and stained with Giemsa solution. For each sample, 200 cells of polychromatic erythrocytes (PCE), the same number of normochromatic erythrocytes (NCE) and the cells containing their micronucleus (MN) were counted using the in vitro MN test. CP increased the frequency of MNPCE and decreased the cell proliferation % (PCE/PCE+NCE). All doses of anthocyanin extract significantly reduced the frequency of MNPCE ($p<0.05$). The cell proliferation ratio (PCE/PCE+NCE) was also enhanced optimally. The most effective dose was 400 mg/kg with O. aspera than O. reticulata i.e., it decreased significantly the frequency of MNPCE and increased the cell proliferation ratio. Results of this study indicated that Osbeckia anthocyanin extract has no genotoxic impact and also potent as anti-mutagenic against cyclophosphamide like compounds induced mutagenicity.

KEY WORDS

Osbeckia aspera and O. reticulata, antimutagenic, micronucleus test, chromosomal aberration, cyclophosphamide.

INTRODUCTION

Osbeckia L. is a genus of the Melastomataceae family. Most of the species of *Osbeckia* are vigorous shrubs which can reach up to 3m. in height and can have a green or reddish stem. It has pink, purple, or purple dotted white flowers, with red or brown fleshy calyces that form the fruits (Kriebel, 2015). It is a tropical plant native to India although it grows widely in the tropical and subtropical regions of both hemispheres (Thabrew *et al*., 1998). The nutritional benefits of the plant were associated with the bioavailability of flavonoids, particularly anthocyanins (Lawarence and Murugan, 2017b).

Therapeutic properties of *Osbeckia* found in the literature include protection of cells from hepatotoxicity induced by carbon tetrachloride.1,2 D-galactosamine, t-butylhydroperoxide and bromobenzene *in vitro* (Pineda *et al*., 1999) The aqueous extract of *Osbeckia* provided

significant protection against paracetamol-induced *in vivo* liver injury, as assessed by histological changes and liver enzyme levels. *In vitro* experiments were also performed to test the ability of the aqueous plant extract to protect freshly-isolated rat hepatocytes against damage produced by 2,6-dimethyl N-acetyl p-quinoneimine (2,6-diMeNAPQI), a stable analogue of the toxic metabolite of paracetamol. Thus, ample evidence state that the aqueous extract of *Osbeckia* has direct protective effects on hepatocytes, and therefore be involved in inducing immune system against viral hepatitis. Antioxidant and immune modulatory effects were also reported with *O. aspera* and *O. wynaadensis.*(Subhasree *et al*., 2009)

Osbeckia octandra was studied for its hepatoprotective activity. Further *O. octandra*, *O. chinensis* and *O. nepalensis* were showed to exhibit anti-diabetic activity (Syiem and Khup, 2006). Despite all these reports, the use of *Osbeckia* fruits and flowers by ethnic people was highly ignored by investigators. *Osbeckia chinensis* fruits were used by the *Meithi* community of Manipur to obtain a medicinally important violet dye and this dye was used in olden days for colouring mouth (Sharma *et al*., 2005). The crushed fruits and flowers were soaked in local made wine or country liquor for best results. The violet dye being anthocyanins, the brightly coloured flowers and fruits also need to be investigated for medicinal properties. Anthocyanins represent one of the most widely distributed classes of plant flavonoids. Apart from their coloring effects, anthocyanins show ability to prevent lipid oxidation, scavenging activity against various artificially generated free radicals. The common aglycon forms of anthocyanidins were cyanidin, delphinidin, peonidin, petunidin, malvidin, and pelargonidin. The prevalent sugar moieties were glucose, rhamnose, xylose, galactose, arabinose, and fructose. A wide variety of phenolic substances, especially those present in food and medicinal plants, have been considered as adjuvants in the prevention of genotoxic effects. Most of these naturally occurring phenolic compounds show antioxidant and anti-inflammatory properties (Wollenweber, 2017).

Substances known as chemo preventive were those capable of inhibiting, slowing, or reversing carcinogenesis at various stages (Wang and Stoner, 2008). Anthocyanins act as antioxidants and are therefore able to fight mutagenic processes (Aswathy *et al*., 2018).

Mutagenesis has become an area of research not only in India, but worldwide. The relevance of this observation was due to the association between mutation and cancer. Ministry of Health data shows that, in 2013, 189,454 people died as a result of malignant neoplasms. For 2016, the occurrence of more than 596,000 cases of the disease in the country was estimated (WHO, 2016; Butelli *et al*., 2017)

According to the World Health Organization (WHO, 2010), obesity and overweight were defined as abnormal or excessive accumulation of fat, derived from a severe metabolic disorder. 52.5% of the adult population in India were overweight. This condition was a health risk linked to serious nutritional problems, which increase the risk of morbidity of various metabolic and chronic diseases among these neoplasms.

Traditional knowledge is an important source of information in locating and obtaining new herbal medicines. An ethnobotanical survey of *Osbeckia* species conducted by Lawarence and Murugan, (2017) in Munnar, Idukki district of Kerala revealed their medical usages by the locals as curative drugs.

According to Pulpra *et al*., (2018) there were still new plant species emerging with the potential to become source of new drugs. However, popular and even traditional use was not enough to validate a plant as herbal medicine, since the lack of reliable information about its properties, adverse reactions, synergistic action, and toxicity potentialities (Agyare *et al*., 2018). In this context, this study aims to evaluate the potentialities of *Osbeckia aspera and O. reticulata* against DNA damage in mice against mutagenicity.

Plant material

Osbeckia sps. for the present study were obtained from various parts of Idukki district of Kerala such as Munnar hills, Wagamon and Peerumedu. *O. aspera,* is a perennial shrub distributed along the tropics and sub tropics. The plant was propagated naturally by vegetative means and seeds. Seed derived plantlets were found to be less frequent in the natural habitat and distributed as fragmented patches. *O. reticulata*, is a small tree seen along the temperate high-altitude habitats i.e., 3000 feet in Western Ghats. *O. aspera* was established in the green house, but *O. reticulata* failed to acclimatize in the greenhouse conditions and remained live up to six months. Identity of the plants

were confirmed by referring floras and authenticated by herbaria of Jawaharlal Nehru Tropical Botanical Garden and Research Institute (JNTBGRI), Palode, Trivandrum.

***In vitro* culture**

The collected plantlets were reared in green house of the college Botanical garden. Stem and leaf cuttings from healthy, disease free plants were used as explants. The explants were subjected to surface sterilisation using 10% teepol solution followed by rinsing in tap water for 60 min. Then, the stem cuttings were immersed in ethanol 70% (v/v) for 30 s followed by mercuric chloride or sodium hypochlorate. Ethanol treatment was found hazardous in the case of leaves. Finally, the explants were washed in autoclaved distilled water.

The sterilized nodal segments (2-3 cm) were used for multiple shoot induction whereas inter nodal fragments and leaf cuttings for direct organogenesis and callus induction. The explants were transferred to MS culture medium supplemented with various phytohormones, besides the control group (without growth regulators). The MS culture media was supplemented with vitamins, sucrose (30 g /L), and agar (7 g /L). The culture media pH was adjusted to 5.7 ± 0.1. Media were sterilized by autoclaving for 15 min at 120°C and 15 lbs of pressure. Clonal fidelity of the regenerated plants was ascertained by morphological and histological evaluation. All the experiments were conducted in a completely randomized design.

Estimation of anthocyanin content

Protocol of Sutharut and Sudarat, (2012) was used for the estimation of anthocyanin content from the flowers. The absorbance was read at 510 and 700 nm against distilled water as blank. Silica gel, Amberlite IRC 80, Amberlite IR 120, DOWEX 50WX8, Amberlite XAD4 and Amberlite XAD7 were trialed for the effective purification of anthocyanins. Aqueous acidified methanol and ethanol were used as the mobile phase for the extraction of anthocyanins.

Animal study

Ten weeks old Swiss albino mice of either sex, weighing 25-30 g, were obtained from M/s Venkateshwara breeders, KLE University's College of Pharmacy, Bangalaru. Mice were maintained under standard environmental parameters (25 ± 1°C, RH 42 ± 5%, light and dark cycle of 12 h), fed with standard pelleted feed and water *ad libitum*. The experimentals were carried as per the ethical guidelines put forth by the MoEF, Government of India (Institutional animal ethical committee Reg. No: PRC/Expt/9/2015-16 dated 24-6-15 and F.No: 25/03/09-AWD, GOI)).

Duration of treatment

Chromosomal aberration analysis on mouse bone marrow cells was carried after 24 h treatment. Analysis of the micronuclei (MN) was conducted in bone marrow cells after 30 h of administration. For combination treatment with cyclophosphoamide (CP), the purified anthocyanin extracts of the *Osbeckia aspera* and *O. reticulata* were given 7 consecutive days prior CP administration.

Design of experiments

Six mice was analyzed from each experimental group, *viz.*, Group I – negative control 0.5 ml distilled water; Group II treatment positive control with 50 mg/kg b.w dose of CP; Group III, IV and V purified anthocyanin extracts from *Osbeckia aspera* and *O. reticulata* (100, 200 and 400 mg/kg, b.w.) followed by 50 mg/kg b. w. of CP dose respectively. All the treatments were given intraperitoneally (i.p) as a single dose.

Analysis of chromosome aberration in bone marrow cells

The controls and experimental mice were injected i.p with 4 mg/kg b.w. of colchicine, as a cytostatic compound, 2 h before the end of the exposure period. After 2 h, the mice were killed by cervical dislocation. The femur bones were dissected out cleanly and the bone marrow was kept in 3 ml of pre-warmed 0.56% KCl solution. The resultant cell suspension was incubated at 36°C for 20 min and the cells were harvested by centrifuging at 600 rpm for 4 min. The pellet of cells was suspended in two drops of KCl solution and was fixed using freshly prepared chilled Carnoy's fluid (3:1 absolute alcohol and glacial acetic acid). The cells were washed with Carnoy's fluid thrice and after the third, the cells were suspended in the fresh fixative. Subsequently, the suspension was placed on a clean pre-chilled slide. Air was blown gently over the slides and warmed for few seconds. Later the slides were stained with 4% giemsa for 12 min followed by rinsing with double distilled deionized water. 200 well-spread metaphase cells were analyzed for chromosomal aberrations from each mice of a treated group. Analysis of slides was carried using oil immersion image analyzer system attached to LEICA DM 500 binocular research microscope (Preston *et al*., 1987) (Sharma and Talukder, 1987)

Statistical analysis

The entire data were evaluated for mean values ± SD for all groups. Statistical comparisons were made using Students *t*-test and $P < 0.05$ was considered significant. The analyses were performed by using the statistical software SPSS.

RESULTS AND DISCUSSION

***In vitro* culture and elucidation of anthocyanin**

In vitro callus cultures were established for the efficient production of anthocyanin from the species *O. aspera* and *O. reticulata.* Although the culture condition requirements were diiferent for the two species, callusing in both species was observed with auxin and cytokinin combinations. Combinations of BA along with NAA and IBA produced compact calli with morphogenesis, meanwhile combination of BA with 2, 4-D produced white friable callus. Combination of either of the cytokinins along with NAA or IBA and higher concentrations of sucrose on the medium (2 to 2.5-fold) induced synthesis of anthocyanin from the friable callus. The highest anthocyanin content in *O. aspera*, was 10.7 mg/g tissue and that for *O. reticulata* was 13.3 mg/g. Anthocyanin content of the callus was found to be higher than the respective flowers of the species.

Purification and fractionation using LC- MS/MS

Anthocyanin was extracted from the calli in water acidified with 1% HCl and purified by column chromatography in silica gel followed by amberlite XAD7HP gel column. LC-MS/MS analysis of purified anthocyanin from *O. aspera* and *O. reticulata* showed significant levels of anthocyanin fractions such as malvidin-3 -diglucoside, peonidin, delphinidin and cyanindin.

Animal study

Swiss albino mice treated with 50 mg/kg b.w CP showed a statistically remarkable increase in the frequency of chromosomal aberrations in the bone marrow cells (Table - 1 &2) as compared to the negative control group ($p < 0.05$). The mutagenicity of CP was further substantiated from the results of micronucleus (MN) test (Fig. 1&2).

Chromosome aberration assay in bone marrow cells

Mutagens like cyclophosphan, diethylnitrosamine, cyclophosphoamide and dimethylnitrosamine etc. interact with the structure of the DNA leads to aberrations via loss, addition, or replacement of bases, thus altering their DNA sequence and the fidelity of the genetic information. *In vivo* assay reveals the efficacy of spectra of changes in chromosomal integrity in metaphase cells and there by chromosomal aberrations. Analysis of metaphase chromosomes from the bone marrow of cells from mice is a proven technique for evaluating the *in vivo* chromosomal damages. The mice bone marrow chromosomal aberration model can identify the clasto or aneugenic effects of a mutagen. Chromosomal aberrations due to the lesions in the DNA leads to discontinuities in the double helix of DNA. Single and double strand breaks, base damage, DNA-DNA and DNA-protein cross bonds, alkylations at base or phosphate molecules, intercalations, thymine dimers, apurinic and apyrimidinic sites in DNA were resulted by mutagenic processes. Failure of repairing the lesions or restoring the original base sequence leads to chromosomal aberrations and gene mutations (Durnev A.D., Kulakova A.V., Zhanataev A.K., 2015). The genotoxicity of four different concentrations of purified anthocyanin (50, 100, 200 and 400 mg/kg b.w.) from *O. aspera*, and *O. reticulata* were analyzed and the results obtained were compared with that of control (Table 1&2). All the four concentrations displayed negligible or insignificant chromosome aberrations in the mice ($P < 0.05$).

Further, pre-treatment with *O. aspera*, and *O. reticulata* exhibited remarkable inhibitory activity against the CP induced chromosomal aberrations (Table 1b, 2b &Fig. 2). Data showed that antimutagenic efficacy of anthocyanin from *O. aspera* was more significant than *O. reticulata* (Fig. 2) i.e., its effect on the blocking of chromosome aberrations was statistically significant as compared to the negative control and CP alone treated groups ($p < 0.05$). Interestingly, the maximum genoprotective effect and the reduced frequency of chromosomal aberrations was recorded with the highest concentrations (200 and 400 mg/kg b.w.) of anthocyanin of *Osbeckia aspera*. *O. reticulata* purified anthocyanin extracts at 400 mg/kg b.w. exhibited inhibitory effect and marginal increase in the frequency of chromosomal aberrations reflecting the insignificant cytotoxic nature of the extracts at the increased doses.

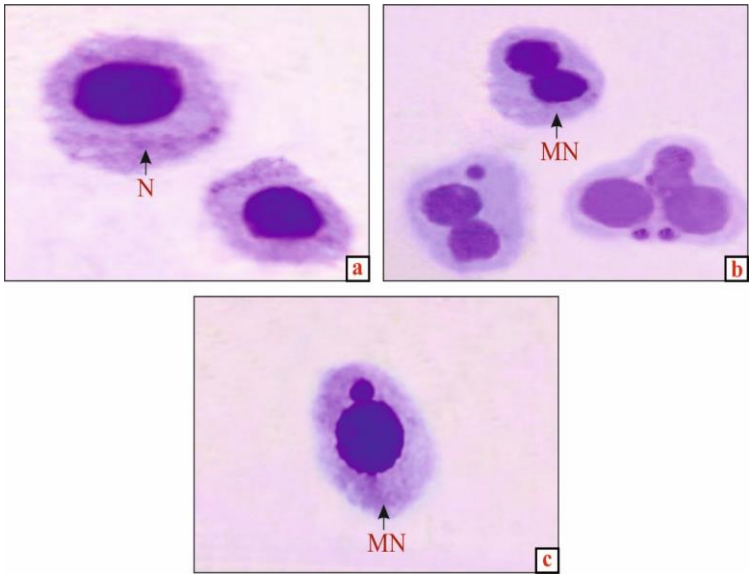

Fig. 1a Normal cells, b and c. micro nucleated (MN) bone marrow cells treated with cyclophosphoamide

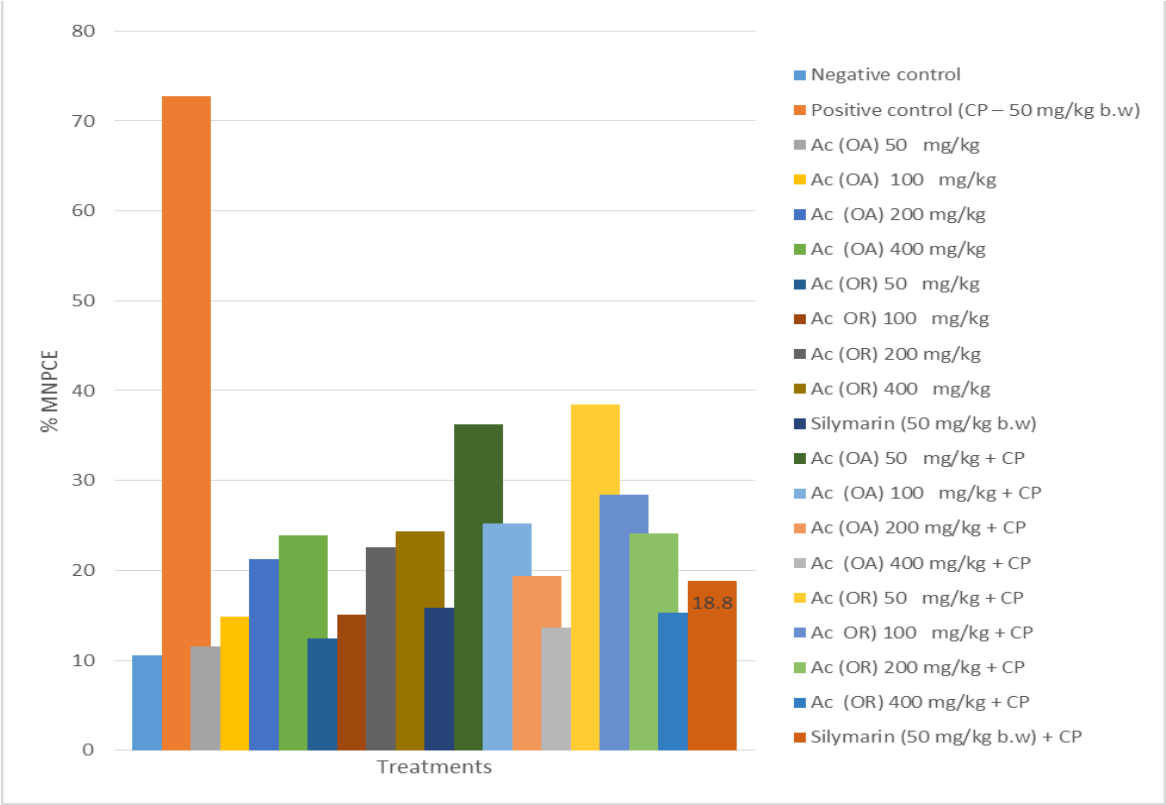

Fig.2: Comparison of changes of micronucleus polychromatic erythrocytes in bone marrow cells of the control group, cyclophosphamide, and varying concentrations of anthocyanin of *O. aspera* and *O. reticulata*

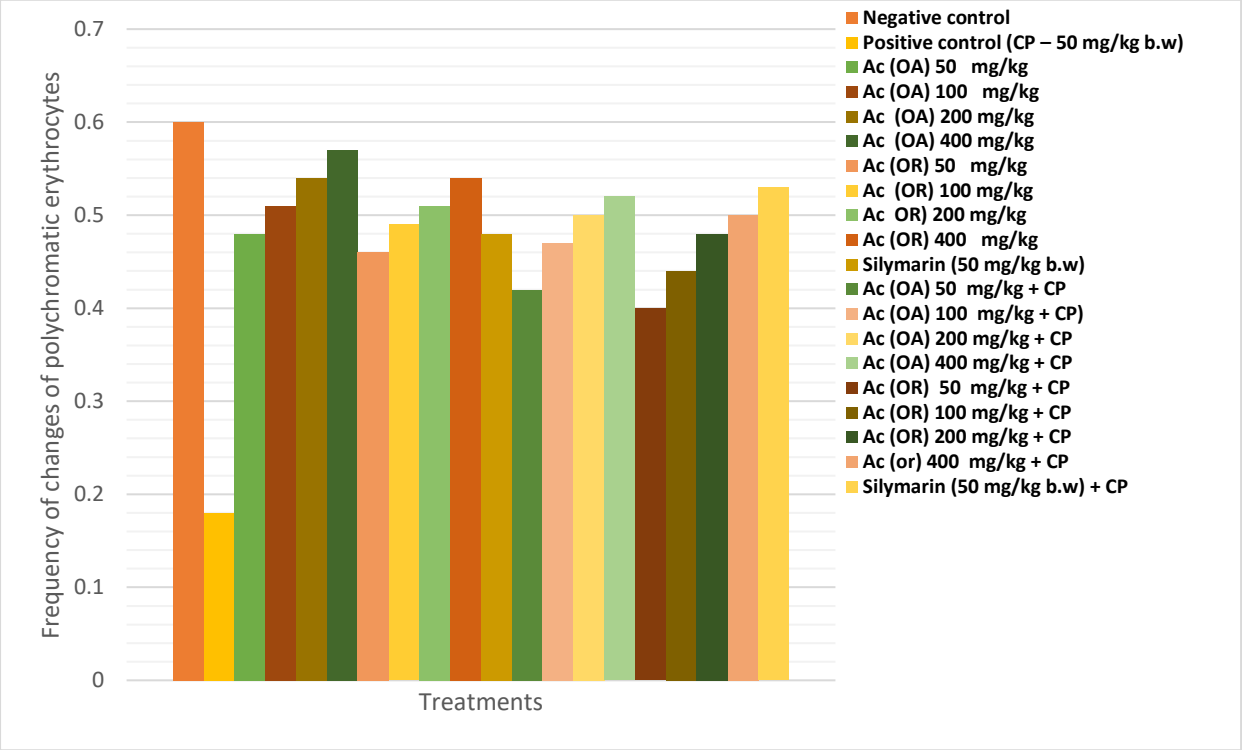

Fig.3: Comparison of Frequency of changes of polychromatic erythrocytes [PCE]/PCE + normal chromatic erythrocytes) in bone marrow cells of the control group, cyclophosphamide, and varying concentrations of anthocyanin of *O. aspera* and *O. reticulata*

Micronucleus (MN) assay in bone marrow cells

Micronuclei originate from fragments or lagging behind of anaphase chromosome or the chromosomes left outside the daughter nuclei in telophase (Sabharwal *et al*., 2015). Thus, the MN test was considered as a powerful technique to analyze clasto and aneugenic impacts of any mutagens. In certain cases, the micronuclei may be originated from fragments originated from broken anaphase bridges formed during the chromosomal rearrangements like dicentric chromatids, intermixed chromosome ring or sister chromatids union(Rasgele *et al*., 2014). Micronuclei were identified in the cells even without the main nucleus in erythrocytes.

The PCE/NCE ratio was also evaluated during the present study. The reduction in PCE/NCE ratio reflects the lagging in the rate of cell division due to the cytotoxic effects of CP. Almeida *et al*., (2017), revealed that an up regulation of NCEs indicates a cytotoxic impact, while an increase in PCEs denotes a stimulation of proliferative potentiality.

Results of micronucleus analysis corroborates with the noticed chromosomal aberrations in the bone marrow cells. Treatment with different concentrations of the anthocyanin extracts of *O. reticulata* and *O. aspera* such as 50, 100, 200 and 400 mg/kg b.w. indicates no significant genotoxic effect on inducing micronuclei in the mice ($P < 0.05$).

Pre-treatment of mice with anthocyanin extracts of *O. reticulata* and *O. aspera* revealed statistically significant decrease in the micronuclei frequency as compared to that of CP treated mice ($p < 0.01$). The reduced frequency of MN formation was also noticed with the treatment of *O. reticulata* at 50, 100 and 200 mg/kg b.w. (Fig. 2). *O. aspera* was more potent even at the highest concentration. The PCE/NCE ratio was recalled back to the level of negative control group (Fig. 3). The highest value for PCE/NCE ratio was showed by *O. reticulata*. The marginal rise in the frequency of micronuclei at the highest dose 400 mg/kg b.w. of *O. reticulata* anthocyanin extracts may be due to the negligible cytotoxic nature of anthocyanin at the higher doses. Generally, antimutagens were nonspecific redox agents, ROSs or free radical scavengers or functions as ligands by binding metals or toxic molecules. Pre-treatment with anthocyanin may induced DNA repair mechanisms and protected them from subsequent exposure to genotoxic agent. The obtained results indicate that pre-

treatment with anthocyanin reduces CP induced mutagenesis in repair proficient mechanism.

Mathew and Thoppil, (2012) compared three *Salvia* extracts and revealed their varied performance of antimutagenic activity. Wongpa *et al*., (2007) analyzed the antimutagenic effects of piperine on CP induced chromosome aberrations in rat bone marrow cells**.** Mohammed, (2017) proved the anti-mutagenic protection of *Quercus infectoria* galls against genotoxicity induced by 2-Aminoanthracene in terms of mice bone marrow mitotic index, chromosomal aberration, and micronucleus assay. 2 g/kg caused the statistically significant decrease of 2AA induced genotoxicity. Słoczyńska *et al*., (2014) reviewed the plausible antimutagenic mechanisms of natural compounds. Ahmad *et al*., (2016) demonstrated the anticarcinogenic and antimutagenic activity of *Alstonia scholaris* on mice bone marrow and peripheral human lymphocyte cells against methyl methane sulfonate induced genotoxicity. Gamal-Eldeen *et al*., (2013) analyzed anti-genotoxic and inhibition of chromosomal aberrations, micronuclei, and DNA fragmentation by the *Sargassum dentifolium*. Zhang *et al*., (2009) blocked cyclophosphamide-induced DNA damage and apoptosis effects of ginsenoside Rb1 on mouse bone marrow cells and peripheral blood leukocytes. Ambasta, (2017) evaluated anticlastogenicity of stem extracts from *Tinospora cordifolia* against arsenic genotoxicity in mice bone marrow erythrocytes using micronucleus assay.

The mechanism of antimutagenic potential by the phytochemicals is still unresolved. Similarly, the genotoxicity is not a carcinogenic measurement but often there is a positive correlation between frequency of micronuclei and the appearance of tumors in mice and humans Vinod *et al*., (2011). Lawarence and Murugan, (2017b) previously proved the antioxidant potential of *Osbeckia* species. Santa-Cecília *et al*., (2012) also correlated antioxidant activity with inflammatory phagocyte superoxide anion release. The mechanism was controlled by protein tyrosine phosphorylation and by the direct stimulation of the protein kinase C.

Negi *et al*., (2003) evaluated the antioxidant and antimutagenic potentialities of pomegranate peel aqueous extracts. Interestingly, low antioxidant activity, with high antimutagenic potentiality. In this context, it is not always needed that antioxidant values are determinants of the antimutagenic potential of products. A study by Santa-Cecília *et al*., (2012) with MI has shown that, even as low antioxidant activity, the MI was able to down regulate inflammatory phagocyte superoxide anion release through a mechanism controlled by protein tyrosine phosphorylation and by the direct stimulation of the protein kinase C.

CONCLUSION

The present study reveals antigenotoxic properties of the purified anthocyanin of selected *Osbeckia* species using bone marrow cells of mice administrated with cyclophosphoamide. *In vitro* calli culture was standardized using culture parameters and phytohormones. Subsequently, the crude anthocyanin was purified using column chromatography followed by fractionation revealed the presence of malvidin-3 - diglucoside, delphinidin, cyanindin aglycone and peonidin. Anthocyanin showed significant arrest of the chromosomal aberrations induced by CP treated mice bone marrow cells. This was further supported by MN assay reduction and enhancement of PCE/NCE ratio. Possibly, the antimutagenic potential may be via the antioxidant activities that might contribute to an anticarcinogenic effect. The overall results suggest that anthocyanins are novel drugs of natural origin as prophylactic antitumor agents.

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Groups	Chromatid aberrations		Chromosome aberrations (ch.ab)						Total Ch. ab
	gap	break	gap	break	del	ring	dc	frag	
Negative control	3.2	5.3	3.2	6.5	5.5	7.3	6.8	5	42.8
Positive control	42	55.7	56.6	45.7	73.3	75.8	70.2	23	442.3
Ac 100 mg/kg	4	7.4	6.4	8	7.3	8	7.5	6.4	55
Ac 200 mg/kg	6.4	8	7.7	10	8. 1	9	10	7	58.1
Ac 400 mg/kg	7.82	9	8.8	11.1	9.34	10	11.2	8.5	75.76

Groups	Chromatid aberrations		Chromosome aberrations (ch.ab)						Total Ch. ab
	gap	break	gap	break	del	ring	dc	frag	
Negative control	3.2	5.3	3.2	6.5	5.5	7.3	6.8	5	42.8
Positive control	42	55.7	56.6	45.7	73.3	75.8	70.2	23	442.3
Ac 100 mg/kg + CP	21.5	30.8	26.9	28.6	33.8	40.6	34.6	12.6	229.4
Ac 200 mg/kg + CP	11.4	16	14.7	14	15.8	22	17	7	117.9
Ac 400 mg/kg + CP	6.2	10.1	8	7.2	10.1	9.7	8.2	5	64.5

Table 1 a & b: *In vivo* chromsomal aberration assay on mice bone marrow cells against different concentration of purified anthocyanin extract from *O. aspera* in comparison with negative and positive (CP) controls($p<0.05$).

Groups	Chromatid aberrations		Chromosome aberrations (ch.ab)						Total ch. ab
	gap	break	gap	break	del	ring	dc	frag	
Negative control	3.2	5.3	3.2	6.5	5.5	7.3	6.8	5	42.8
Positive control	42	55.7	56.6	45.7	73.3	75.8	70.2	23	442.3
Ac 100 mg/kg	5.8	9.6	8.4	9.7	8.8	9.3	9.2	7.6	68.4
Ac 200 mg/kg	7.6	10.1	9.5	11.7	10.5	11.5	12.2	9.3	82.4
Ac 400 mg/kg	9.2	11.4	10.9	13.2	12	13.2	13.6	10	93.5

Groups	Chromatid aberrations		Chromosome aberrations (ch.ab)						Total ch.ab
	gap	break	gap	break	del	ring	dc	frag	
Negative control	3.2	5.3	3.2	6.5	5.5	7.3	6.8	5	42.8
Positive control	42	55.7	56.6	45.7	73.3	75.8	70.2	23	442.3
Ac 100 mg/kg + CP	20.7	32.1	29.2	30	34.5	38.6	37.6	13.4	236.1
Ac 200 mg/kg + CP	12.4	17.3	15.2	15.6	16.4	23.5	18.2	8.3	126.9
Ac 400 mg/kg + CP	7.1	11.3	9.4	8.8	11.2	12	9.8	9.6	79.2

Table 2 a & b*: In vivo* chromsomal aberration assay on mouse bone marrow cells against different concentration of purified anthocyanin extract from *O. reticulata* in comparison with negative and positive (CP) controls

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